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The selection between apoptosis and necrosis is differentially regulated in hydrogen peroxide-treated and glutathione-depleted human promonocytic cells

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Abstract

Treatment with 0.2 mM hydrogen peroxide (H_2O_2) or with 0.5 mM cisplatin caused caspase-9 and caspase-3 activation and death by apoptosis in U-937 human promonocytic cells. However, treatment with 2 mM H_2O_2, or incubation with the glutathione suppressor DL-buthionine-(*S,R*)-sulfoximine (BSO) prior to treatment with cisplatin, suppressed caspase activation and changed the mode of death to necrosis. Treatment with 2 mM H_2O_2 caused a great decrease in the intracellular ATP level, which was partially prevented by 3-aminobenzamide (3-ABA). Correspondingly, 3-ABA restored the activation of caspases and the execution of apoptosis. By contrast, BSO plus cisplatin did not decrease the ATP levels, and the generation of necrosis by this treatment was not affected by 3-ABA. On the other hand, while all apoptosis-inducing treatments and treatment with 2 mM H_2O_2 caused Bax translocation from the cytosol to mitochondria as well as cytochrome *c* release from mitochondria to the cytosol, treatment with BSO plus cisplatin did not. Treatment with cisplatin alone caused Bid cleavage, while BSO plus cisplatin as well as 0.2 and 2 mM H_2O_2 did not. Bcl-2 overexpression reduced the generation of necrosis by H_2O_2, but not by BSO plus cisplatin. These results indicate the existence of different apoptosis/necrosis regulatory mechanisms in promonocytic cells subjected to different forms of oxidative stress.
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Keywords: apoptosis; necrosis; H_2O_2; cisplatin; glutathione depletion; promonocytic cells

Abbreviations: 3-ABA, 3-aminobenzamide; BSO, DL-buthionine-(*S,R*)-sulfoximine; cisplatin, CDDP, *cis*-platinum(II)-diammine dichloride; FCS, fetal calf serum; GSH, reduced glutathione; H_2O_2, hydrogen peroxide; mAb, monoclonal antibody; pAb, polyclonal antibody; PARP, poly (ADP-ribose) polymerase; PBS, phosphate-buffered saline; PCD, programmed cell death; PI, propidium iodide; PKC, protein kinase C; ROS, reactive oxygen species

Introduction

Apoptosis and necrosis were originally described as the two alternative forms of cell death, with well-defined morphological and biochemical differences. Apoptosis, often considered as equivalent to 'programmed cell death' (PCD), was described as the regulated, 'physiological' type of death by which the organism eliminates senescent, abnormal and potentially harmful cells. By contrast, necrosis was described as a passive, nonphysiological type of death caused by cytotoxic insults.[1] However, this general picture became less clear later because (i) there are forms of PCD with intermediate characteristics between genuine apoptosis and necrosis[2,3] and (ii) also because cytotoxic agents (such as antitumour drugs, radiation, oxidants and hyperthermia) may provoke cell death with characteristics of apoptosis.[4–6] In these cases, the mode of death may be often experimentally selected by modifying the conditions of treatment.[5,7,8] In practical terms, apoptosis (or other forms of apoptosis-like PCD)[3] is clearly advantageous for the organism since it leads to the elimination of the dying cells by phagocytosis, thus preventing the release of intracellular content and the damage of the surrounding tissue. Hence, it seems very important to elucidate the mechanisms that ultimately decide the adoption of one or the other mode of death.

A factor that may trigger cell death, and that may decide the selection between apoptosis and necrosis, is the increase in intracellular oxidation. Using treatment with exogenous hydrogen peroxide (H_2O_2) as an experimental paradigm of oxidation, it was often observed that moderate H_2O_2 concentrations triggered apoptosis, while elevated concentrations caused necrosis (the exact concentrations depending very much on the used cell model).[5,9–11] Moreover, the application of a low concentration of exogenous H_2O_2 sufficed to suppress apoptosis and cause necrosis-like death in Burkitt's lymphoma cells treated with antitumour drugs.[11] In this model, the change in the mode of death was attributed to the oxidant-mediated depletion of intracellular ATP, which is a strict requirement for the execution of apoptosis. However, excessive oxidation might also suppress apoptosis by other mechanisms, such as direct inactivation of caspases.[10,12] An alternative method to cause oxidation is by decreasing the intracellular concentration of antioxidant molecules, making the cell unable to cope with the normal production of reactive oxygen species (ROS). In this manner, the depletion of intracellular reduced glutathione (GSH) by prolonged incubation with DL-buthionine-(*S,R*)-sulfoximine (BSO), a specific inhibitor of γ-glutamylcysteine synthetase, caused an in-

crease in the intracellular content of H_2O_2, and potentiated the increase provoked by other treatments.[13,14] Correspondingly, the incubation with BSO potentiated the lethality of cytotoxic treatments,[13–15] leading in some cases, to a change of the mode of death from apoptosis to necrosis.[7,16]

We have recently reported that the alkylating drug *cis*-platinum(II)-diammine dichloride (cisplatin, CDDP), which normally caused death by apoptosis, provoked necrosis when applied to U-937 promonocytic cells with a reduced GSH content.[8] Although necrosis induction was accompanied by an increased intracellular concentration of peroxides, and could be prevented by antioxidants, it remained unclear whether the change in the mode of death could be adequately explained as the simple consequence of increased ROS accumulation. As a further investigation, in the present work we comparatively analyse some mechanisms critical for the regulation of cell death in U-937 cells subjected to the above-described models of oxidation, namely treatment with exogenous H_2O_2, and treatment with cisplatin with and without GSH suppression. The results demonstrate that, among other differences, the switch from apoptosis to necrosis in H_2O_2-treated cells is the consequence of ATP depletion, while in BSO plus cisplatin-treated cells it is independent of ATP, and apparently associated with the inhibition of mitochondrial cytochrome *c* release.

Results

Death induction and caspase activities

Figure 1 compares the generation of apoptosis and necrosis in U-937 cells treated with different concentrations of H_2O_2, and with cisplatin with and without preincubation with BSO. For quantification, the rate of apoptosis was determined by the frequency of cells with condensed/fragmented chromatin (Figure 1a) and with sub-G_1 (hypodiploid) DNA content

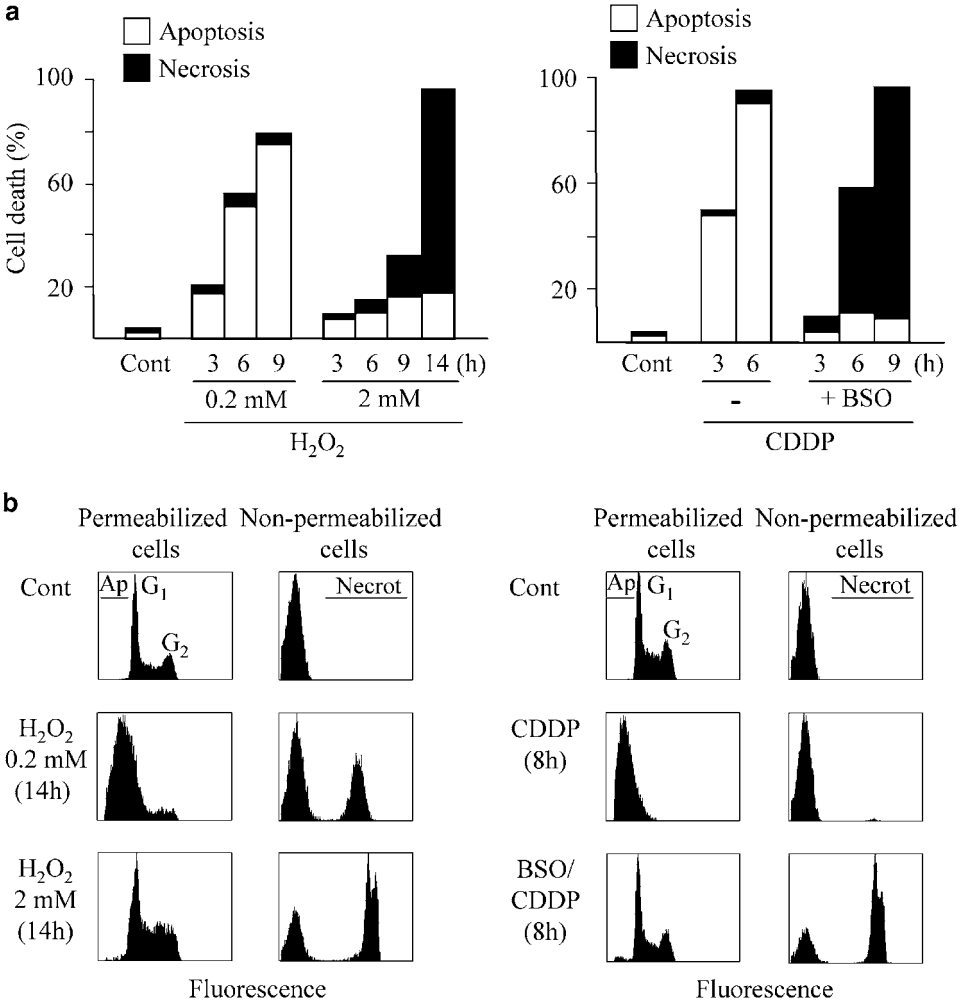

Figure 1 Generation of apoptosis and necrosis by H_2O_2 and cisplatin. (**a**) Frequency of apoptotic and necrotic cells, as determined by chromatin fragmentation and trypan blue permeability, respectively, in untreated cultures (Cont), in cultures treated for the indicated time periods with the indicated concentrations of H_2O_2 and in cultures treated for the indicated time periods with 0.5 mM cisplatin (CDDP), either in the absence (−) or the presence of BSO. BSO (1 mM) was applied 24 h before treatment with cisplatin. (**b**) Cell distribution according to PI incorporation into cultures subjected to the indicated treatments, as revealed by flow cytometry. An aliquot of each culture was subjected to permeabilisation (left row in each group), to show the cell cycle distribution and the fraction of apoptotic (sub-G1) cells (Ap), while another aliquot was processed without prior permeabilisation (right row in each group), to determine the fraction of cells which allowed free PI uptake (necrotic). The histograms are representative of one of three (**a**) or two (**b**) experiments, with similar results

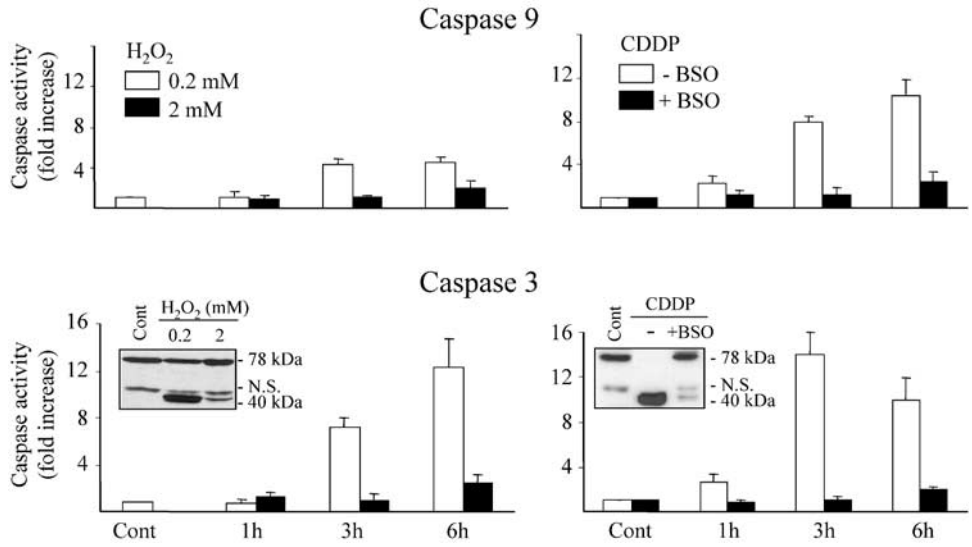

Figure 2 Stimulation of caspase activities. Total cellular extracts (10 μg protein per sample) obtained from untreated cells and from cells subjected to the indicated treatments were used to determine the caspase-9 and caspase-3 activities, using as substrates LEHD-*p*NA and DEVD-*p*NA, respectively. The results (mean $\pm$ S.D. of three determinations) are represented in relation to untreated cells, which was given the arbitrary value of one. (Insets) PKCδ cleavage, as determined by immunoblotting, at 6 h of treatment. The upper band corresponds to the whole protein (approximately 78 kDa), and the lower band to the main cleavage fragment (approximately 40 kDa). All other conditions were as in Figure 1

(Figure 1b). The rate of necrosis was determined by the frequency of cells with free penetration of trypan blue (Figure 1a) or propidium iodide (PI) (Figure 1b), indicating plasma membrane damage. In both models, apoptotic cells presented surface blebbing and reduction in size, while necrotic cells exhibited normal chromatin distribution (similar to untreated cells) and cell swelling (results not shown). It was observed that treatment with 0.2 mM H_2O_2 only caused apoptosis, while the concentration of 2 mM preferentially caused necrosis. As an exception, the fraction of cells characterised as necrotic in the 0.2 mM H_2O_2 treatment in Figure 1b probably represents apoptosis-derived 'secondary' necrosis, because of the length of the treatment (14 h). In these experiments, the execution of necrosis experienced a 5–6 h delay in relation to the execution of apoptosis (see Figure 1a). In addition, it was observed that treatment with 0.5 mM cisplatin alone caused death by apoptosis, but the mode of death was almost totally diverted to necrosis if the treatment was preceded by a 24 h preincubation with 1 mM BSO. Under these conditions the execution of apoptosis was more rapid, and the delay of necrosis in relation to apoptosis lower (approximately 2–3 h) than in the case of treatments with H_2O_2. These differences could not be avoided, since increasing the H_2O_2 concentration (from 0.2 to 0.4 mM, and from 2 to 6 mM) did not substantially accelerate the execution of apoptosis and necrosis; and decreasing the cisplatin concentration to 0.05 or 0.1 mM delayed apoptosis, but in this case the combination of BSO plus cisplatin did not produce necrosis (results not shown). A detailed description of other effects of cisplatin and BSO, such as peroxide accumulation, GSH depletion, and changes in mitochondrial transmembrane potential, was already presented in a preceding work,[8] and hence is omitted here.

The capacity of H_2O_2 and cisplatin to cause apoptosis or necrosis, depending on the conditions of treatment, correlated with their capacity to activate the caspase-9/caspase-3 cascade, required for the execution of the 'intrinsic' pathway of apoptosis (the pathway commonly activated by cytotoxic treatments).[17] Thus, extracts from cells treated with 0.2 mM H_2O_2 or cisplatin alone exhibited a marked increase in LEHDase (indicative of caspase-9) and DEVDase (indicative of caspase-3) activities, while these activities were much lower in extracts from cells treated with 2 mM H_2O_2 and BSO plus cisplatin (Figure 2). These results were corroborated by the observation that the apoptosis-inducing treatments elicited the typical caspase-3-mediated protein kinase c (PKC)δ cleavage to give a fragment of approximately 40 kDa,[18] while the cleavage was null or very poor in the case of the necrosis-inducing treatments (see insets in Figure 2).

ATP levels

Apoptosis is an energy-dependent process, in such a manner that the decrease of ATP below critical levels may impede the execution of apoptosis and promote necrosis.[19,20] For this reason, experiments were carried out to compare the fluctuations of ATP levels upon treatment with H_2O_2 and cisplatin, both under apoptosis and necrosis-inducing conditions. The treatments were carried out for a maximum of 3 h, to prevent possible ATP leakage through the damaged plasma membranes in necrotic cells. The results are indicated in Figure 3. Treatments with 0.2 mM H_2O_2 and with cisplatin alone caused a slight decrease in the ATP level, which at 3 h reached levels of 60–75% of the control value. Treatment with 2 mM H_2O_2 caused a rapid and profound depletion of ATP, which at 1 h reached levels of around 10% in relation to control cells. By contrast, BSO plus cisplatin did not decrease, and even augmented the ATP content.

To corroborate the different roles of ATP for the regulation of apoptosis/necrosis induction in the two assayed models,

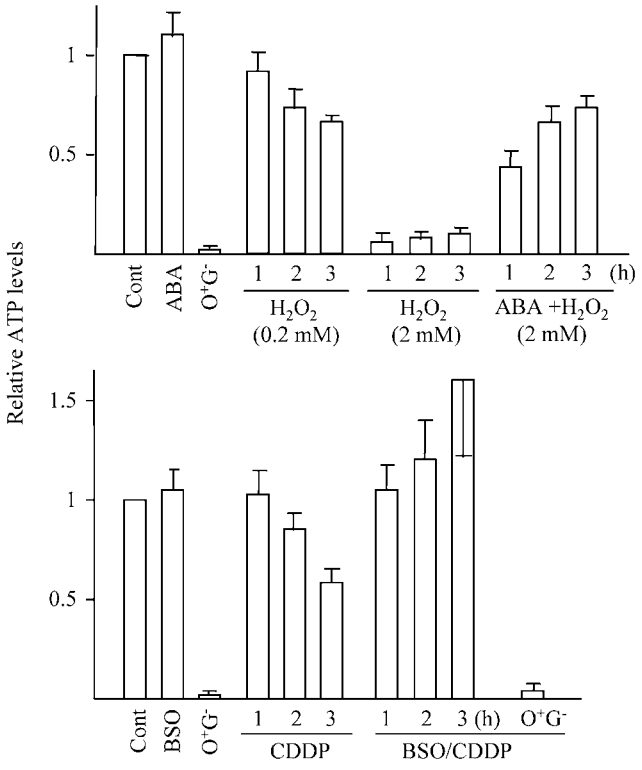

Figure 3 Modulation of ATP levels. The upper histogram shows the relative ATP content in extracts from untreated cells (Cont), from cells treated for 3 h with 3-ABA alone, and from cells treated for the indicated time periods with 0.2 and 2 mM H_2O_2, with and without 3-ABA. The lower histogram shows the relative ATP content in extracts from cells treated with BSO alone, and from cells treated for the indicated time periods with cisplatin, with and without BSO. 3-ABA (2.5 mM) was added 30 min before H_2O_2. To control the accuracy of the technique, in some experiments the cells were incubated for 3 h in glucose-free medium supplemented with 10 μM oligomycin (O^+G^-), to suppress all sources of ATP production. The results are the mean $\pm$ S.D. of at least three determinations. The ATP content in untreated cells was (24.2 ± 3.1 nmol/10^6 cells). All other conditions were as in Figure 1

we examined the effects of the poly (ADP-ribose) polymerase (PARP) inhibitor 3-aminobenzamide (3-ABA). This agent was reported to preserve the ATP pool in cells treated with DNA-damaging agents, preventing the generation of necrosis derived from severe ATP depletion.[21,22] In agreement with this, we found that 3-ABA partially restored the ATP content in cells treated with 2 mM H_2O_2, which in this case maintained a level of at least 40% in relation to control cells (Figure 3). The elevation of ATP levels was accompanied by the suppression of necrosis and the restoration of apoptosis as the mode of death (Figure 4a), and correspondingly by the restoration of the capacity to stimulate caspase-9 and caspase-3 activities (Figure 4b). By contrast, 3-ABA did not switch back the mode of death from necrosis to apoptosis nor restore caspase activities in BSO plus cisplatin-treated cells (Figure 4), which as indicated above failed to decrease the intracellular ATP content.

Cytochrome *c* release

The release of the cytochrome *c* from the mitochondria to the cytosol is required for the assembly of the apoptosome and hence for the activation of the caspase cascade in the intrinsic pathway of apoptosis.[17] However, cytochrome *c* release is not necessarily a distinctive feature of apoptosis, since it was also occasionally observed in association with necrosis.[23–26] For these reasons, immunoblot assays were carried out to detect the presence of cytochrome *c* in cytosolic extracts from cells treated with H_2O_2 and cisplatin, under both apoptosis- and necrosis-inducing conditions. As indicated in Figure 5a, cytochrome *c* could be detected in extracts from cells treated with both 0.2 and 2 mM H_2O_2, which caused apoptosis and necrosis, respectively. By contrast, cytochrome *c* was only detected in extracts from cells treated with cisplatin alone, which caused apoptosis, but not in extracts from cells treated with BSO plus cisplatin, which caused necrosis. The results obtained with cisplatin were confirmed by examining the subcellular distribution of cytochrome *c* and the mitochondrial bc_1 complex, by means of immunofluorescence microscopy. Thus, untreated cells and cells treated with BSO plus cisplatin (necrotic) mostly exhibited yellow fluorescence, resulting from the overlapping of the green (indicative of bc_1) and red (indicative of cytochrome *c*) signals. By contrast, cells treated with cisplatin alone (apoptotic) clearly exhibited segregated areas of red fluorescence, indicating the presence of cytochrome *c* outside the mitochondria (Figure 5b).

Expression of Bcl-2 protein family members

The release of cytochrome *c* is regulated by proteins of the Bcl-2 family, which may either inhibit (e.g. the antiapoptotic proteins Bcl-2 and Bcl-X_L) or promote (e.g. the proapoptotic protein Bax) the process.[27] Bax is normally present as an inactive monomer in the cytosol, and to be functional requires oligomerisation and translocation to the mitochondrial membrane.[27] In addition, cisplatin and other cytotoxic drugs may stimulate Bid cleavage and insertion into the mitochondrial membrane, where it also promotes cytochrome *c* release.[28,29] For these reasons, immunoblot assays were carried out to measure the level, integrity and subcellular distribution of Bcl-2, Bcl-X_L, Bax and Bid. The results, indicated in Figure 6, were as follows: (i) Bcl-2, Bcl-X_L and Bax were constitutively expressed in untreated cells, and their total levels were little affected by treatment with H_2O_2 and cisplatin, both under apoptosis- and necrosis-inducing conditions (Figure 6a). (ii) In untreated cells, Bax was mostly detected in the cytosolic fraction and only a minimal part in the mitochondrial fraction. Treatment with 0.2 and 2 mM H_2O_2 and with cisplatin alone, which as indicated above provoked cytochrome *c* release, caused a progressive increase (from 2 to 3 h) in Bax levels in mitochondrial extracts. By contrast, there was no increase in mitochondrial extracts from cells treated with BSO plus cisplatin, which failed to cause cytochrome *c* release (Figure 6b). Bcl-2 was always undetectable in cytosolic fractions (result not shown), and its level in the mitochondrial fractions was not significantly modified by the treatments, being used as an internal control (Figure 6b). (iii) Treatment with cisplatin alone caused a progressive loss (from 2 to 3 h) of Bid proform (21 kDa), while treatments with BSO plus cisplatin and with 0.2 and 2 mM H_2O_2 did not (Figure 6c). In spite of the commercial specifications of the used antibodies, it was almost impossible to detect Bid fragments by Western blot.

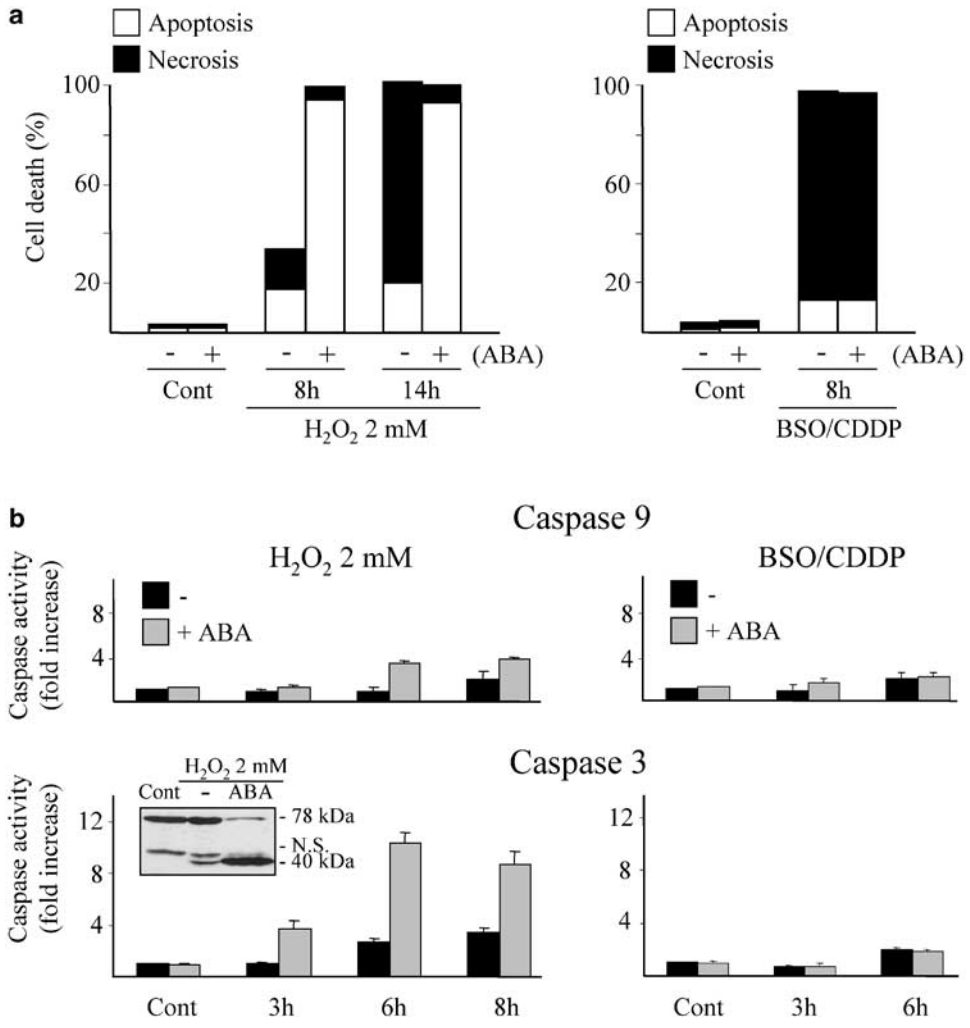

Figure 4 Modulation of cell death and caspase activities by 3-ABA. (**a**) The histograms show the frequency of apoptotic and necrotic cells, as determined by chromatin fragmentation and trypan blue permeability, respectively, in untreated cultures (Cont) and in cultures treated for the indicated time periods with 2 mM H_2O_2 or with BSO plus cisplatin, either in the absence (−) or the presence (+) of 3-ABA. (**b**) The histograms show the caspase-9 and caspase-3 activities in untreated cells (Cont), in cells treated for 6 h with 3-ABA alone; and in cells treated for the indicated time periods with 2 mM H_2O_2 or with BSO plus cisplatin, with or without 3-ABA. All other conditions were as in Figures 1–3

Hence, we consider the loss of Bid proform as an indication of cleavage, a criterion also used by other authors.[30,31]

In addition to the well-known action of Bcl-2 as an antiapoptotic factor, some reports indicate that Bcl-2 overexpression may also inhibit necrosis.[32–34] For this reason, experiments using Bcl-2-transfected U-937 cells were carried out to determine the effect of Bcl-2 overexpression on the generation of necrosis by 2 mM H_2O_2 and BSO plus cisplatin. Our control experiments revealed an approximately eight-fold increase in Bcl-2 content in transfected *versus* nontransfected cells (result not shown). The results are indicated in Figure 7. It was observed that Bcl-2 overexpression decreased the frequency of necrosis following H_2O_2 treatment. Of note, this represented an effective cell protection, since the lower frequency of necrosis was not compensated by an increase in the frequency of apoptosis. By contrast, Bcl-2 overexpression did not protect, and even slightly accelerated the generation of necrosis by BSO plus cisplatin. As expected, Bcl-2 overexpression greatly inhibited apoptosis induction by treatment with 0.2 mM H_2O_2 and cisplatin alone (result not shown).

Discussion

The results in this work corroborate earlier observations indicating that H_2O_2 causes apoptosis or necrosis, depending on the concentration used,[5,9,10] and that the depletion of intracellular GSH diverts the mode of death from apoptosis to necrosis in cells treated with cisplatin and other alkylating agents.[8,16] Since one of the main functions of GSH is to act as a ROS scavenger, GSH depletion may cause *per se* an overaccumulation of H_2O_2 and/or potentiate its accumulation by treatment with cytotoxic agents, as we had in fact demonstrated to occur in the case of cisplatin.[8] Hence, one could hypothesise that the mechanism(s) that regulate cell death upon treatment with BSO plus cisplatin are the same one(s) operating upon treatment with high H_2O_2

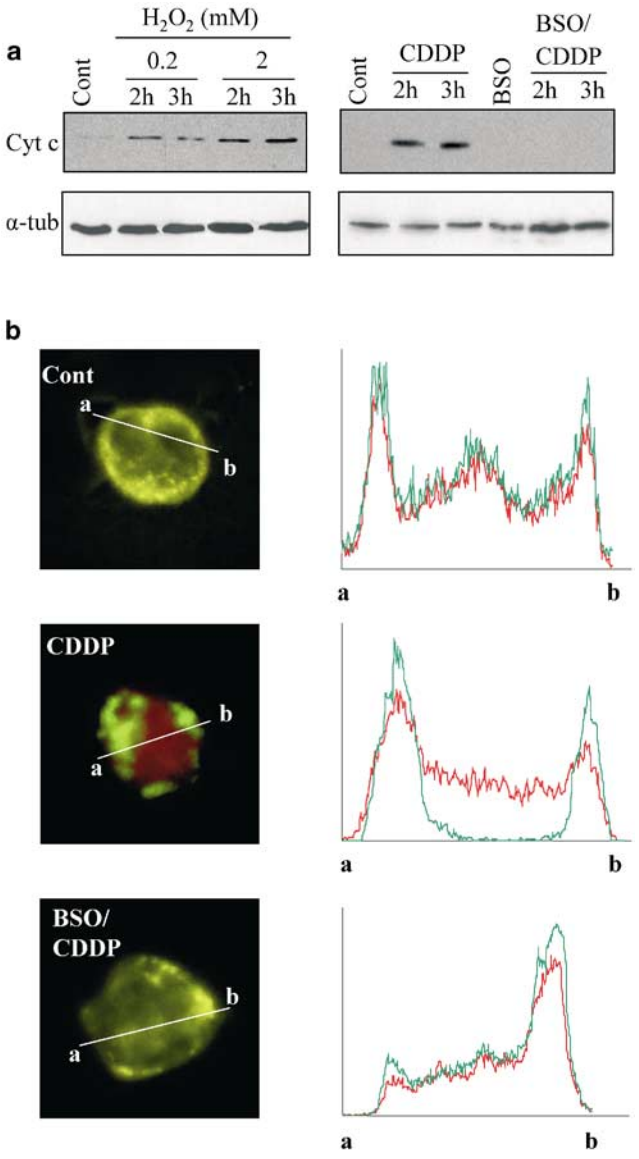

Figure 5 Cytochrome *c* release. (**a**) Cytosolic extracts (25 μg protein per lane) obtained from untreated cells (Cont), from cells incubated with BSO alone (BSO), from cells treated for the indicated time periods with the indicated concentrations of H_2O_2 and from cells treated for the indicated time periods with cisplatin, with or with BSO, were used to measure the relative level of cytochrome *c* by means of immunoblotting. The level of α-tubulin was also measured as a control. The blots are representative of one of four experiments with similar results. (**b**) Untreated cells (Cont), and cells treated for 3 h with cisplatin, with or without BSO, were fixed and processed for immunolocalisation of cytochrome c and mitochondrial bc_1 complex. The yellow fluorescence indicates colocalisation of bc_1 (green signal) and cytochrome *c* (red signal), while the red fluorescence in CDDP indicates segregation of cytochrome *c*. The graphs indicate the distribution of fluorescence in sections of the cells (the orientation given by a and b). All other conditions were as in Figure 1

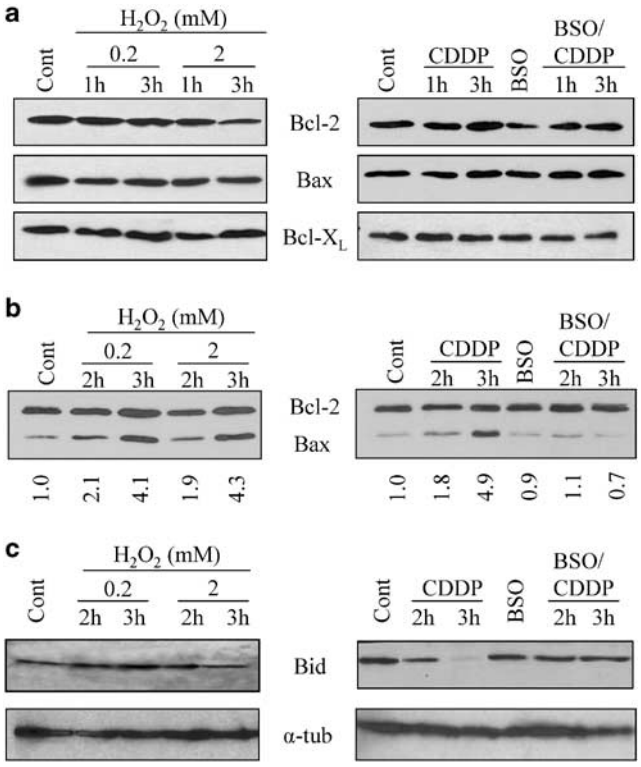

Figure 6 Expression and processing of Bcl-2 protein family members. Extracts obtained from untreated cells (Cont), from cells incubated with BSO alone (BSO), from cells treated for the indicated time periods with the indicated concentrations of H_2O_2 and from cells treated for the indicated time periods with cisplatin, alone or with BSO, were used for immunoblot assays. (**a**) Relative levels Bcl-2, Bax and Bcl-X_L in total cellular extracts. (**b**) Relative levels of Bcl-2 and Bax in mitochondrial extracts. The numbers at the bottom of each lane represent the corresponding Bax/Bcl-2 ratio, as determined by densitometry, in relation to the control value. (**c**) Relative levels of Bid in total cellular extracts. Samples corresponding to 15, 15 and 30 μg protein per lane were used in A, B and C, respectively. The determinations were repeated twice in (**a**) and at least three times in (**b** and **c**), with similar results. All other conditions were as in Figure 1

concentrations. Actually, both treatments were unable to activate the caspase-9/3 cascade, characteristic of the mitochondrial pathway of apoptosis. However, the mechanisms responsible for caspase inhibition, and hence for the suppression of apoptosis, are not coincident. Thus, while 2 mM H_2O_2 greatly decreased the intracellular ATP content, but allowed cytochrome *c* release to the cytosol, BSO plus cisplatin suppressed cytochrome *c* release, but did not decrease ATP.

The inability of 2 mM H_2O_2 to cause apoptosis may be explained by the sharp reduction in ATP levels, which impedes the assembly of the apoptosome and hence caspase activation.[17] In addition, the lack of ATP could directly cause necrosis by preventing the functioning of a plasma membrane-associated Na^+/K^+ ATPase normally active during apoptosis, with the consequence of ionic unbalance, water uptake, and cell swelling and lysis.[35] The minimum content of intracellular ATP compatible with the execution of apoptosis has been estimated in approximately 25% of the normal value.[20] Our results fit with this estimation, since (i) 0.2 mM H_2O_2 only caused a slight (approximately 25%) reduction in ATP levels and generated apoptosis; (ii) 2 mM H_2O_2 reduced the ATP level by approximately 90% and caused necrosis and (iii) 3-ABA restored the ATP level to approximately 40–50% of control values and restored apoptosis. These latter results could be explained by changes in the activity of PARP, a molecule recently characterised as a critical switch between apoptosis and necrosis.[36] Thus, the oxidative stress caused by elevated H_2O_2 concentrations may generate extensive

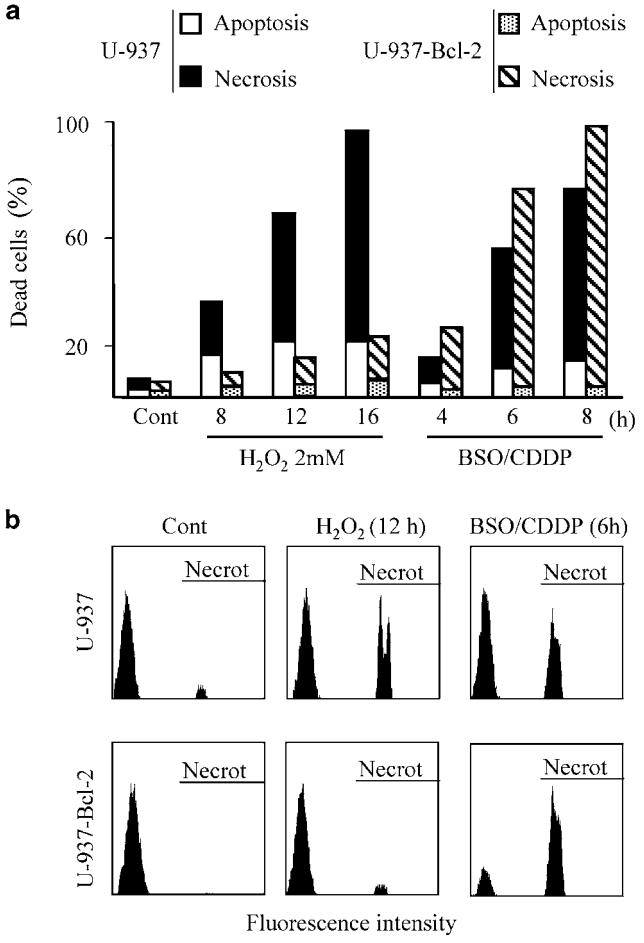

Figure 7 Effect of Bcl-2 overexpression on the generation necrosis. (**a**) Frequency of apoptotic and necrotic cells, as determined by chromatin fragmentation and trypan blue permeability, respectively, in nontransfected (U-937) and Bcl-2-transfected (U-937-Bcl-2) cell cultures treated for the indicated time periods with the 2 mM H_2O_2 or with BSO plus cisplatin. (**b**) Frequency of necrotic cells using the same cell types and treatments, as revealed by PI penetration and flow cytometry. The values were repeated three times (**a**) and twice (**b**) with similar results. All other conditions were as in Figure 1

DNA damage, leading to PARP activation, NAD^+ consumption and massive ATP depletion.[37] Under these conditions, the administration of 3-ABA may inhibit PARP activation, partially preserving the ATP pool. Alternatively or complementarily, H_2O_2 might suppress the *de novo* ATP synthesis by inhibiting mitochondrial respiration and/or glycolytic enzyme activities;[38] and 3-ABA could preserve ATP by preventing the block of glycolysis.[39] Whatever the case, it seems clear that the generation of necrosis by BSO plus cisplatin is independent of ATP, as far as the treatment did not reduce the ATP level, and necrosis was not prevented by 3-ABA.

Another manner in which H_2O_2 could suppress apoptosis is by direct inactivation of caspases, because of the oxidation of the cysteine residues in their catalytic sites[10] (although this mechanism has been challenged).[40] This was apparently the case in menadione-treated HepG2 cells, where menadione increased oxidation, decreased ATP levels and caused necrosis, but necrosis could not be prevented by the elevation of ATP.[12] However, this mechanism does not seem to operate in our model, since the increase of ATP levels by 3-ABA sufficed to restore caspase activities and apoptosis. Of note, we and others have reported that the administration of the pan-caspase inhibitor Z-VAD-Fmk prevented the execution of apoptosis in U-937 cells.[8,41,42] However, in this case the suppression of apoptosis was not accompanied by cell swelling, and plasma membrane integrity was only affected at approximately 24 h (i.e. much later than in our present experiments). This may be in agreement with reports indicating that caspase inhibition *per se* might be insufficient to cause classical necrosis.[3] Hence, the generation of necrosis under oxidant conditions (treatment with H_2O_2 or GSH depletion) probably involves additional physiological disturbances, for example, ionic unbalance, as indicated above.

The inability of BSO plus cisplatin to activate the caspase cascade and hence to cause apoptosis may be adequately explained by the lack of cytochrome *c* release from mitochondria to the cytosol; and the lack of cytochrome *c* release could be the consequence, at least in part, of the lack of Bax translocation to mitochondria. Actually, there was always a correlation between Bax translocation and cytochrome *c* release in the two types of treatments used, H_2O_2 and cisplatin. In addition, Bid might participate in the regulation of cytochrome *c* release in cisplatin-treated cells, since cisplatin alone caused Bid cleavage (as deduced from the disappearance of the 21 kDa proform), while BSO plus cisplatin did not. Noteworthy, H_2O_2 failed to stimulate Bid processing under all used conditions, a result also reported by other authors.[31] The disparity in the behaviour of Bid might indicate that, in addition to necrosis, apoptosis is also differentially regulated in cells subjected to H_2O_2 and cisplatin. Finally, although no attempts were made to examine the influence of Bcl-2 expression on cytochrome *c* release, the experiments using Bcl-2-transfected cells lead to interesting results. On the one hand, Bcl-2 overexpression reduced the generation of both apoptosis and necrosis by H_2O_2. This was not surprising, since one of the earlier described actions of Bcl-2 is to activate antioxidant functions in the cell,[43,44] and other reports also indicated inhibition by Bcl-2 of necrosis induction by H_2O_2 and other cytotoxic treatments.[32–34] By contrast, Bcl-2 overexpression did not reduce, and even facilitated the generation of necrosis by BSO plus cisplatin. This result was unexpected, since Bcl-2 was reported to counteract the increase in toxicity generated by GSH depletion.[44,45] A full understanding of these results requires a deeper investigation, but meanwhile they reinforce the conclusion of a different regulation of necrosis induction by H_2O_2 and BSO plus cisplatin.

Although one of the roles of GSH is the scavenging of peroxides, GSH also regulates other important aspects of the redox equilibrium. For instance, glutathione may form reversible mixed disulphides with protein thiols, preventing irreversible oxidation of cysteine residues under oxidative stress.[46] Hence, the lack of GSH upon prolonged treatment with BSO could compromise this defensive function. In addition, the lack of GSH may result in an increase in the intracellular free, acting concentration of alkylating drugs, since these drugs are detoxified by GSH conjugation.[47] This is also important, since cisplatin is a very reactive molecule, which directly binds nucleophilic sites of many cellular components, including proteins.[48] In these ways, BSO plus

cisplatin could severely alter the function of proteins critical for apoptosis–necrosis regulation – such as Bcl-2 family members (as demonstrated here in the case of Bax) and/or mitochondrial permeability transition pore constituents.[49] This might explain the existence of substantial differences in the regulation of cell death by different forms of oxidative stress – namely, direct treatment with H_2O_2 *versus* GSH depletion.

Materials and Methods

Chemicals

All components for cell culture were obtained from Life Technologies, Inc. (Gaithersburg, MD, USA). 4,6-diamidino-2-phenylindole was obtained from Serva (Heidelberg, Germany); RNase A from Roche Diagnostics, S.L. (Barcelona, Spain); digitonin, caspase-3 substrate I, (Ac-DEVD-*p*NA) and caspase-9 substrate II (LEHD-*p*NA) from Calbiochem (Darmstadt, Germany); and 3-ABA, BSO, DL-dithiothreitol, cisplatin, oligomycin, PI and protease inhibitor cocktail from Sigma (Madrid, Spain). Mouse anti-human Bcl-2 (100) monoclonal antibody (mAb), rabbit anti-human Bax (N-20) polyclonal antibody (pAb), and rabbit anti-human nPKCδ (C-20) pAb were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Rabbit anti-human Bcl-X_L pAb was from Transduction Laboratories (Lexington, KY, USA). Mouse anti-pigeon cytochrome *c* mAb (clone 7H8.2C12, used for immunoblot assays) and mouse anti-rat cytochrome *c* mAb (clone 6H2.B4, used for immunofluorescence microscopy) were from BD PharMingen (San Diego, CA, USA). Goat anti-human Bid (C-20) and goat anti-mouse Bid pAbs were obtained from Santa Cruz Biotechnology and R&D Systems, Inc. (Wiesbaden-Nordenstad, Germany). Mouse anti-human COX II was obtained from Molecular Probes (Eugene, OR, USA). Rabbit anti-rat bc_1 complex was a generous gift of Professor R Bisson, Padova, Italy. Mouse anti-chicken α-tubulin mAb, fluorescein isothiocyanate-conjugated goat anti-rabbit IgG, and tetramethylrhodamine isothiocyanate-conjugated goat anti-mouse IgG were from Sigma. Peroxidase-conjugated rabbit anti-mouse IgG and peroxidase-conjugated goat anti-rabbit IgG were from DAKO Diagnósticos, S.A. (Barcelona, Spain).

Cell culture and treatments

U-937 human promonocytic leukemia cells,[50] and Bcl-2-transfected U-937 cells (kindly provided by Dr. Jacqueline Bréard, INSERM 461, Chatenay Malabry, France) were grown in RPMI 1640 supplemented with 10% (v/v) heat-inactivated fetal calf serum (FCS), and 0.2% sodium bicarbonate and antibiotics in a humidified 5% CO_2 atmosphere at 37°C. 4,6-diamidino-2-phenylindole (10 mg/ml) and PI (1 mg/ml) were dissolved in phosphate-buffered saline (PBS), and cisplatin (3.3 mM) in distilled water. These solutions were stored at 4°C. 3-ABA (100 mM) was dissolved in distilled water and stored at −20°C. BSO (50 mM) was freshly prepared in distilled water, just before use. Typically, the cells were seeded at 2×10^5 cells/ml 24 h before treatment with cisplatin or H_2O_2. For GSH depletion, the cells were incubated for 24 h with 1 mM BSO, before treatment with cisplatin. Under these conditions BSO caused an approximately 70% reduction in total GSH content, but did not affect cell proliferation or viability. To obtain a complete ATP depletion, the cells were incubated in the presence of 10 μM oligomycin in glucose-free RPMI medium, supplemented with 1 mM sodium pyruvate and 10% (v/v) dialysed FCS.

Determination of apoptosis and necrosis

To analyse changes in nuclear morphology, the cells were collected by centrifugation, washed with PBS, resuspended in PBS and mounted on glass slides. After fixation in 70% (v/v) ethanol, the cells were stained for 20 min at room temperature in PBS containing 1 μg/ml 4,6-diamidino-2-phenylindole and examined by fluorescence microscopy. Apoptosis was characterised by chromatin condensation followed by partition into multiple bodies. Within the experimental time periods used in this work, nonapoptotic, primary necrotic cells still exhibited diffuse and uniform chromatin staining, as untreated cells. To measure loss of DNA, cells were collected by centrifugation and incubated for 30 min in PBS containing 0.5 mg/ml RNase A and 0.1% (w/v) Nonidet P-40. After the addition of PI (final concentration of 50 μg/ml), the cells were analysed by flow cytometry. Late apoptotic cells exhibited sub-G_1 PI incorporation (hypodiploid cells). Within the experimental time periods used in this work, nonapoptotic, primary necrotic cells did not exhibit significant loss of DNA, nor significant alterations in the cell cycle distribution in relation to untreated cells.

The criterion currently used to examine necrosis was the loss of membrane integrity, as measured by massive influx of either trypan blue or PI in nonpermeabilised cells. In the first case, cells were incubated for 5 min with 0.2% (w/v) trypan blue and examined by microscopy using a Neubauer haemacytometer. Under these conditions, only necrotic cells were clearly stained. In the second case, nonpermeabilised cells were suspended in PBS containing 50 μg/ml PI, and the fluorescence was analysed by flow cytometry. Under these conditions only necrotic cells exhibited great fluorescence, while the fluorescence was null or very low in apoptotic cells.

Caspase activity assays

Samples of 4×10^6 cells were collected by centrifugation, washed twice with ice-cold PBS, resuspended in 50 μl of ice-cold lysis buffer (1 mM DL-dithiothreitol, 0.03% Nonidet P-40 (v/v), in 50 mM Tris pH 7.5), kept on ice for 30 min and finally centrifuged at 14 000 $\times$ *g* for 15 min at 4°C. Samples containing aliquots of the supernatants (corresponding to 10 μg of total protein), 8 μl of the appropriate caspase substrate (Ac-DEVD-*p*NA for caspase-3, and LEHD-*p*NA for caspase-9), and PBS to complete 200 μl, were prepared by triplicate in 96-well microtiter plates, and incubated for 1 h at 37°C. The absorption was measured by spectrometry at 405 nm.

Measurement of ATP levels

To estimate the intracellular ATP content, aliquots of 2×10^6 cells were collected in a preheated (70°C) buffer consisting of 100 mM Tris and 4 mM EDTA, pH 8, and heated for 2 min at 100°C. After cooling on ice and centrifugation at 10 000 $\times$ *g* for 1 min at 4°C, the ATP content in the supernatants was determined using an ATP Bioluminescence Assay Kit CLSII (Roche Diagnostics, Barcelona, Spain), following the procedure indicated by the manufacturer, and a TD-20/29 luminometer (Turner Designs, Sunnyvale, CA, USA). ATP standard curves (linear in the range of 5–500 nM) were carried out in all experiments. Extracts from cells depleted of ATP by incubation with oligomycin-containing glucose-free medium were routinely used as control of the technique.

Protein extraction, subcellular fractionation and immunoblot assays

To obtain total cellular protein extracts, samples of 3×10^6 cells were collected by centrifugation, washed with PBS and lysed by 5 min heating at 100°C in Laemmli's buffer containing protease inhibitors followed by sonication. To obtain cytosolic extracts for the determination of

cytochrome *c* release, samples of 3×10^6 cells were collected by centrifugation, and permeabilised for 5 min in 100 μl of ice-cold PBS containing 80 mM KCl, 250 mM sucrose and 200 μg/ml digitonin. After centrifugation, the pellet was discarded and the supernatant used for immunoblotting. Control determinations using anti-COX II antibody revealed that the supernatants were free of mitochondrial contamination. To obtain mitochondrial and cytosolic extracts for the determination of Bax localisation, the subcellular fractionation procedure described by Kim *et al*.[51] was followed, with minor modifications. Samples of 10^7 cells were washed with PBS and resuspended in 300 μl of solution A (20 mM HEPES–KOH, pH.7.5, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, supplemented with 250 mM sucrose and protease inhibitors). The cells were lysed by passing them five times through a 25-gauge needle, after which the homogenates were centrifuged for 10 min at 1000 *g* at 4°C to remove unbroken cells, nuclei and large membrane fragments. The upper part of the supernatant (approximately 200 μl) was carefully removed and centrifuged for 20 min at 10 000 $\times$ *g* at 4°C. The resulting pellet (mitochondrial fraction) was washed with solution A, centrifuged again, and solubilised by heating and sonication in Lammli's buffer containing protease inhibitors. The resulting supernatant was centrifuged for 1 h at 100 000 *g* at 4°C to obtain the cytosolic fraction. Control experiments using anti-COX II and anti-α-tubulin antibodies were carried out to check the purity of mitochondrial and cytosolic fractions. In all cases, the detection of specific proteins either in the total cellular extracts or in the cell fractions was carried out by immunoblot, following the previously described procedure.[52]

Immunofluorescence microscopy

U-937 cells immobilised on slides were processed for simultaneous detection of cytochrome *c* and bc_1, following the earlier described procedure.[53]

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